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(57) Abstract

Increased expression of the antigen CD45RO on circulating B cells has been identified as a marker of Crohn's disease and multiple sclerosis.

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MARKER FOR CROHN'S DISEASE AND MULTIPLE SCLEROSIS

The invention relates to the detection of Crohn's disease and multiple sclerosis by means of a B cell marker.

Background of the Invention

Crohn's disease is a systemic immunoregulatory disorder, with primarily intestinal manifestations. Although the etiology of this disease is poorly understood, an increase in intestinal permeability has been suggested as a key initiating event (Hollander et al. (1986), Ann. Int. Med., v. 105, pp. 883-885).

No convenient blood test has been available, however, to identify patients suffering from Crohn's disease and to distinguish this disease from other diseases such as ulcerative colitis and celiac sprue, which give rise to very similar clinical presentations (Hamilton, S.R., in "Pathology of the Colon, Small Intestine and Anus", Ed. Norris, H.T. (1983)).

Clinically, the discrimination of these diseases is important because prognosis, complications and management of these patients differs significantly. Additionally, the existence of a discriminating immune marker for Crohn's disease which can be monitored in blood might well offer insight into the fundamental disorder of systemic immunoregulation exhibited by (IBD) patients. Furthermore, new drug therapies could be assessed by such an objective test since clinical improvement may be followed by such a marker, in conjunction with the more subjective Crohn's Disease Activity Index. Until now, there has been a lack of such markers for patients suffering from IBD.

The present inventors have identified a B-cell differentiation antigen or marker which provides a useful marker for Crohn's disease. Presence of this marker permits differentiation of Crohn's disease from ulcerative colitis and celiac sprue by means of a simple, rapid blood test.

This B-cell marker has also been found to be useful as a means of monitoring disease progression and predicting likely severity in Crohn's disease patients.

Examination of relatives of patients suffering from Crohn's disease has also revealed that this B-cell marker is the basis of a convenient blood test for pre-clinical Crohn's disease. Presence of this marker in relatives shows a correlation with above-normal intestinal permeability.

Multiple sclerosis (MS) is a disease involving neurologic inflammation of unknown etiology. Crohn's disease and MS are epidemiologically related, with inflammatory bowel disease patients being forty time more likely than the general population to acquire MS (Minuk et al., New. Eng. J. Med., v. 314, Feb. 27, 1986, p. 586).

No convenient blood test has so far been available to identify MS in patients presenting with early stage symptoms.

The present inventors have found that a B-cell differentiation antigen linked to Crohn's disease is also useful as a marker for MS.

Summary of the Invention

In accordance with one aspect of the present invention, there is provided a method for detecting Crohn's disease in a human subject comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of the antigen being indicative of Crohn's disease.

In accordance with another aspect of the present invention, there is provided a method for

distinguishing Crohn's disease from ulcerative colitis and celiac sprue in a human subject presenting with intestinal symptoms, comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of the antigen being indicative of Crohn's disease.

In accordance with another aspect of the present invention, there is provided a method for detecting pre-clinical Crohn's disease in a human subject, comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of the antigen being indicative of pre-clinical Crohn's disease.

In accordance with another aspect of the present invention, there is provided a method for detecting multiple sclerosis or pre-clinical multiple sclerosis in a human subject, comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of the antigen being indicative of multiple sclerosis or pre-clinical multiple sclerosis.

In accordance with a further aspect of the present invention, there is provided a kit for detecting

Crohn's disease or pre-clinical Crohn's disease in a human subject, comprising

(1) a first antibody specific for a B cell antigen;

(2) a second antibody specific for the CD45 isoform, CD45RO; and

(3) reagent means for detecting the binding of the antibodies to peripheral B cells.

Summary of the Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1A shows CD45RA expression on $CD19^+$ PBMC B-cells from normal subjects, Figure 1B from patients with ulcerative colitis, Figure 1C from celiac sprue and Figure 1D from Crohn's disease.

Files were gated for $CD19^+$ cells and the distribution of CD45 isoform plotted. The vertical axis denotes cell number and the horizontal axis, log fluorescence intensity.

Figure 2A shows CD45RO expression on $CD19^+$ PBMC B-cells from normal subjects, Figure 2B from patients with ulcerative colitis, Figure 2C from celiac sprue and Figure 2D from Crohn's disease. Axes as in Figure 1.

Figure 3 shows a plot of the percentage of $CD19^+CD45RA^+$ or $CD19^+CD45RO^+$ B-cells in Crohn's disease, ulcerative colitis and normal PBMC.

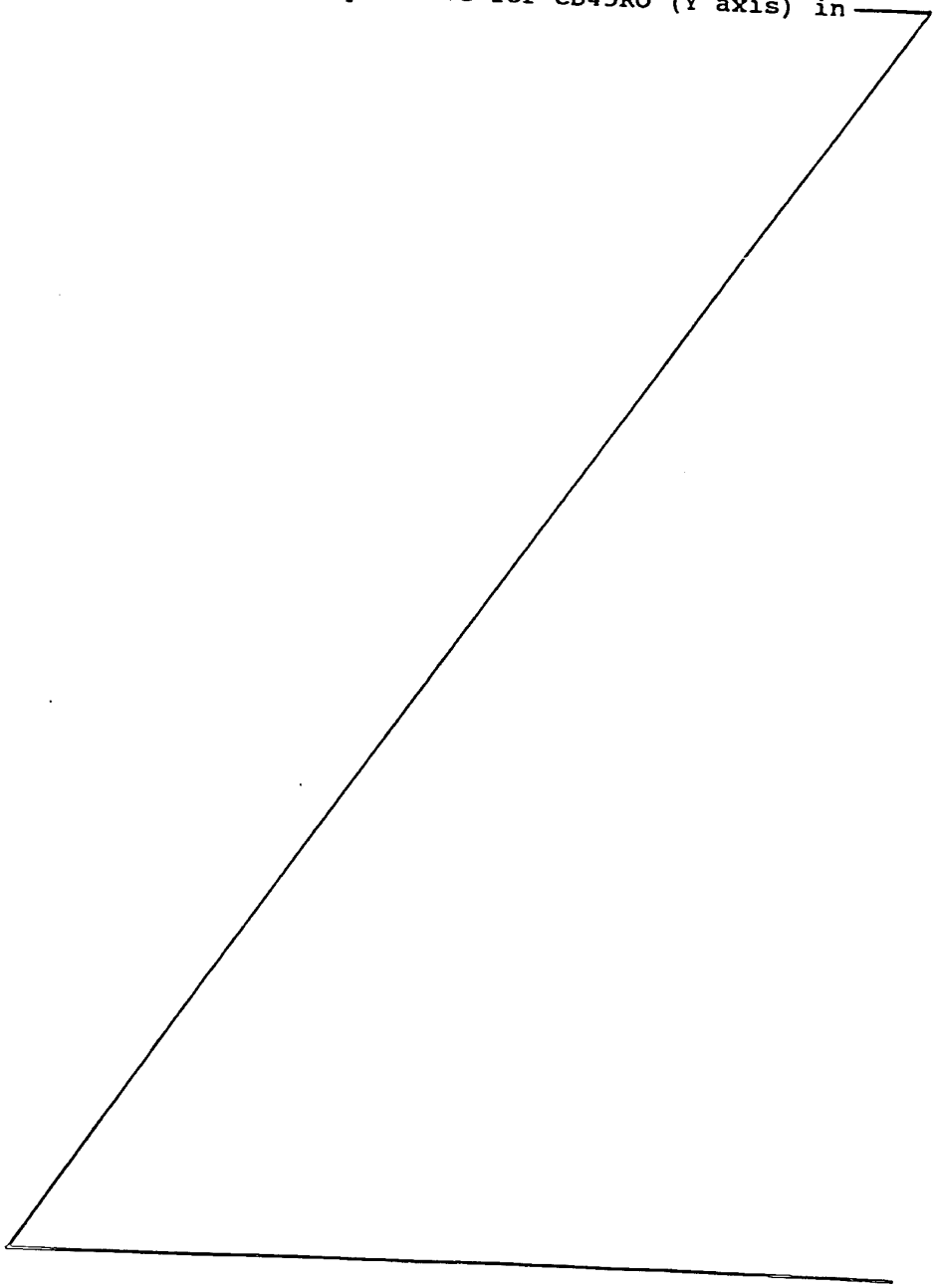
Figure 4 shows $CD45RA^-RO^+$ expression on $CD19^+$ PBMC B-cells from patients with Crohn's disease (X axis) versus Crohn's Disease Activity Index (CDAI: Y axis) for same patients.

Figure 5 shows intestinal permeability, expressed as lactulose/mannitol excretion ratio (Y axis) of indicated groups of subjects (X axis).

Figure 6 shows the percentage of $CD19^+$ peripheral B-cells positive for CD45RO (Y axis) in

Crohn's disease patients with normal or high intestinal permeability.

Figure 7 shows the percentage of $CD19^+$ peripheral B-cells positive for CD45RO (Y axis) in



relatives of Crohn's disease patients with normal or high intestinal permeability.

Detailed Description of the Invention

Specific aberrations of the mucosal immune system have been identified in inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis.

For instance, a predominance of $CD4^+$ and lymphokine activated killer lymphocytes (LAK) together with a relative deficiency of $CD8^+$ cells has been found among Crohn's lamina propria lymphocytes (LPL) (James et al., (1986), Gastroenterology, v. 91, p. 1483). This same study identified a decrease in $CD45RA^+$ $CD4^+$ in LPL as compared to peripheral blood mononuclear cells (PBMC). LPL from patients with IBD have been found to have heightened expression of activation markers (including transferrin receptor, IL-2 receptor and 4F2) (Pallone et al., (1987), Gut, v. 28, p. 745; Schreiber et al., (1991), v. 101, p. 1020).

Humoral immune abnormalities in IBD involve various autoantibodies including rheumatoid factor and anti-colon antibodies disputedly secondary to the underlying mucosal inflammation and not a primary pathophysiologic factor (Pallone et al., (1986), J. Clin. Lab. Immunol., (1986), v. 19, p. 175; Vecchi et al., (1988), in "Inflammatory Bowel Disease: Current Status and Future Approach", Ed. MacDermott, R.P., Excerpta Medica, 1988, pp. 455 - 460).

None of these studies disclosed any conveniently assayed peripheral blood markers indicative of IBD.

The present inventors have analysed the phenotypes of B and T cells from patients with various IBD's, with an emphasis on changes in the B-cells populating the lamina propria in comparison to those in peripheral blood nuclear cells (PBMC) and on the

selective expression of CD45 isoforms as a marker for differentiation within the B-cell lineage. Also analysed were CD5 and CD11b which have been associated with autoimmune B-cells or the activation status of the B-cell (Marcos et al., (1988), Immunol. Today, v. 9, pp. 204-207). CD45, the leukocyte common antigen, is the most prevalent antigen on the surface of B and T lymphocytes and through its cytoplasmic domain, the tyrosine phosphatase activity plays a key role in intracellular signalling (Thomas, M.L., (1989), Ann. Rev. Immunol., v. 7, p. 339). The CD45 isoforms, CD45RA and CD45RO are distinguished by differences in molecular weight, glycosylations and are encoded by alternatively spliced mRNA.

These isoforms characterise T-cell differentiation with the transition from expression of high molecular weight CD45RA (p220) isoform on naive cells to the low molecular weight CD45RO (p180) isoform on memory T-cells. A recent study of this group of cell surface molecules in normal human intestinal mucosal $CD3^+$ T-cells has shown that the intraepithelial lymphocyte (IEL) population expressed mainly CD45RO (Brandtzaeg et al., (1989), Scand. J. Immunol., v. 30, p. 123). $CD3^+$ $CD45RO^+$ T-cells are probable memory T-cells consistent with their role as primary immune regulators of an antigen-bombarded environment such as the gut. However, B-cells are also found among the mucosal lymphocytes in normal and disease states. A transition in CD45 isoforms similar to that found in T-cells has been found in B cells (Marcos et al., (1988), Immunol. Today, (1988), v. 9, pp. 204-207; Jensen et al., (1989), Int. Immunol., v. 1, p. 229). Pre-B cells express exclusively CD45RA at low density, with an increase in CD45RA density as differentiation proceeds towards mature B-cell function. A transition from CD45RA to CD45RO expression appears to occur on <u>in vivo</u> antigen stimulated B-cells which has been confirmed by <u>in vitro</u> studies. Early plasma cells

express only the low molecular weight CD45 isoforms, while end stage plasma cells eventually lose all CD45 expression (Jensen et al., supra).

The present inventors have examined CD45 isoforms in both lamina propria lymphocytes (LPL) and peripheral blood mononuclear cells (PBMC), both T-cells and B-cells, in normal subjects and in patients suffering from Crohn's disease, ulcerative colitis and celiac sprue. Crohn's PBMC were studied by multiple regression analysis in relation to a number of clinical parameters corresponding to patients studied. Cells were probed with differently labelled antibodies to CD45RA and CD45RO. The patterns of CD45 isoform expression on $CD3^+$ T-cells from mucosal LPL were similar to normal in Crohn's disease or UC, with a preponderance of $CD45RA^-RO^+$ cells (normals 66% ± 8%) as shown in Table 1, third column).

In contrast, when $CD3^+$ T-cells from peripheral blood lymphocytes were examined, a significantly increased level of $CD45RA^-RO^-$ cells were found (16% as seen in Table 2, fourth column), and this increase was not seen in UC or celiac sprue.

No significant differences from normal were noted for the percentage of $CD45RA^+RO^-$, $CD45RA^+RO^+$ or $CD45RO^-RA^+$ lymphocytes in Crohn's disease, UC or celiac sprue.

A range of CD45 isoforms were identified on $CD19^+$ B-cells from mucosal LPL. As a group, UC, Crohn's and normal LPL $CD19^+$ B-cells included more cells bearing a transitional pattern of CD45 isoform expression (RA^+RO^+) or bearing neither isoform (RA^-RO^-) than are found among circulating B-cells (Table 3). Among normal LPL, the $CD45RA^+$ RO^- phenotype was detected on 45% ± 5% of $CD19^+$ B lymphocytes, identifying a mature resting B-cell population; 50-66% of B-cells in IBD and normal LPL expressed CD45RO, which appears on activated and late stage B-cells. Expression of CD45RO was found on 23% of

normal LPL B-cells, consistent with definition as a late stage B/pre-plasma cell. Coexpression of both CD45RA and CD45RO, consistent with definition as an activated B-cell, was found on 27% of normal LPL B-cells (Table 3, Line 3, 2nd and 3rd columns). Five percent of normal $CD19^+$ LPL lacked both CD45RA and RO.

When $CD19^+$ B-cells from peripheral blood mononuclear cells were examined, it was found that cells from normals and from UC and celiac sprue expressed the $CD45RA^+RO^-$ isoform almost exclusively, as seen in Table 4, first column.

In contrast, PBMC from patients with Crohn's disease included 44% of B-cells with an abnormal phenotype, either lacking expression of CD45RA, or coexpressing CD45RA and CD45RO (Table 4, Line 1). Fifteen percent of $CD19^+$ PBMC B-cells of patients with Crohn's disease expressed $CD45RA^-RO^+$, 13% coexpressed both CD45RA and RO, and 16% were $CD45RA^-RO^-$. Thus, these B-cells have a CD45 isoform distribution consistent with their being a population of late stage antigen-activated B lymphocytes.

Non-malignant $CD45RO^+$ $CD19^+$ PBMC have so far been identified only after <u>in vitro</u> stimulation or in PBMC from patients with multiple myeloma, Waldenstrom's macroglobulinemia and chronic lymphocytic leukemia (Streuli et al., <u>supra</u>; Jensen et al., (1991), Am. J. Hoematol., v. 37, p. 20; Maddy et al., (1989), Immunol., v. 68, pp. 346 - 352). They have never previously been observed in Crohn's disease.

These findings are the basis of a new method for diagnosing Crohn's disease and for discriminating between Crohn's disease on the one hand and UC or celiac sprue on the other.

The expression of CD45 isoforms on B-cells from Crohn's disease PBMC was very heterogeneous, unlike that from normal donors, UC or celiac sprue. Figure 1 shows the consistently high and relatively uniform CD45RA

density on normal, UC and celiac sprue B-cells in contrast to the broad density distribution on PBMC B-cells from Crohn's disease. Crohn's B-cells included a clearly $CD45RA^-$ negative population and a broad distribution of $CD45RA^+$ cells. A similar degree of heterogeneity was evident for the CD45RO expression on B-cells (Figure 2). PBMC from Crohn's patients included a bimodal but heterogeneous population of $CD45RO^+$ B-cells with predominantly high antigen density (Figure 2). In general, those cells with a low intensity of CD45RO were those cells coexpressing CD45RA (Figure 1 and Table 4).

As seen in Example 4, no increase of CD5 or CD11b expression was found on $CD19^+$ B-cell PBMC from Crohn's disease patients, suggesting that the isolation procedures have not led to activation of the B-cells.

$CD45RA^+RO^-$ as well as RA^-RO^- subset phenotypes were represented among $CD19^+$ PBMC from patients with Crohn's disease in contrast to UC or normal PBMC. This pattern of $CD45RO^+$ and transitional isoform expression suggests a population of activated or late stage B-cells as might be predicted if they play a role in the general heightened immune response associated with Crohn's disease. The heterogeneity of CD45 expression on $CD19^+$ PBMC from patients with Crohn's disease was statistically studied with a number of clinical parameters (including the Crohn's disease activity index (Best, W.R. et a., (1976), Gastroenterology, v. 70, pp. 439-444), disease duration, extraintestinal disease, disease location (colon, small bowel, colon and small bowel), erythrocyte sedimentation rate, medication and age of patient) by multiple regression analysis to determine if any of these variables could account for the aberrant pattern of CD45 isoform expression found on this population of PBMC. Of these clinical parameters, only the CDAI was found to be statistically correlated with high significance with the $CD45RA^-RO^+$ subpopulation of $CD19^+$ PBMC in Crohn's disease.

After grouping high and low CD45RO expression on $CD19^+$ B-cells, a significant statistical correlation was found between these and high CDAI and low CDAI respectively.

This indicates that the isoform pattern detected by the present inventors is a good indication of clinical status in Crohn's disease, as well as serving to discriminate between Crohn's disease and other gut diseases such as celiac sprue and UC. The provision of such an indication of clinical status gives clinicians a convenient method of monitoring disease status in Crohn's patients, based on a simple blood sample and without invasive surgery or gut biopsies. One possible explanation of these results is that the correlation of $CD45RA^-RO^+$ $CD19^+$PBMC with the CDAI results from a stimulated immune system in more severely ill patients either as a primary event initiating intestinal inflammation or as part of an inflammatory cascade once inflammation has been initiated. CD5 and CD11b were absent from LPL or PBMC B-cells, suggesting that these represent late stage B-cells, possibly memory cells, rather than cells in the process of activation which would express both of these antigens based on patterns of activation in vitro.

In accordance with one embodiment of the present invention, a method is provided for detecting Crohn's disease in a human subject and for distinguishing Crohn's disease from ulcerative colitis and celiac sprue in human subjects presenting with clinical symptoms which would be associated with any of these diseases.

The method comprises obtaining a blood sample from the subject, isolating or identifying a sample of B cells from the blood sample and examining the B cells for expression of the CD45 isoform designated CD45RO. The presence of increased CD45RO on peripheral blood B cells is indicative of Crohn's disease. Where potentially confusing intestinal symptoms are present, the presence

of increased CD45RO on B cells points to Crohn's disease and excludes ulcerative colitis and celiac sprue.

Up to about 4 to 5% of circulating B cells in normal subjects are positive for CD45RO. A percentage of CD45RO - positive B cells greater than about 4 to 5 indicates increased and abnormal expression of this isoform.

In accordance with a preferred embodiment of the invention, peripheral blood mononuclear cells are prepared from the subject's blood sample and contacted with a first antibody bearing a first label, the antibody being specific for a B cell antigen and with a second antibody bearing a second label detectable separately from the first label, the second antibody being specific for the antigen CD45RO.

Detection of the first label permits identification of the B cells and these are examined for the presence of the second label, indicative of CD45RO expression. One suitable means of carrying out this examination is by flow cytometry of the labelled cells, where the labels are immunofluorescent labels.

As will be understood by those skilled in the art, the first antibody may be specific for any antigen which identifies or characterises B cells. Anti-CD19 or anti-CD20 antibodies are preferred.

As will also be understood, a great variety of antibody labels are suitable, as long as the first and second labels can be separately identified and determined. For example, the first label may be FITC and the second label may be PE.

In accordance with a further embodiment, the antibodies used to bind to B cell antigen and to CD45RO may themselves be unlabelled and may be rendered detectable after binding by coupling to a signal generating moiety. For example, the bound antibody may be coupled with detectable label or may be coupled to one member of a signal generating pair, such as anti-

immunoglobulin biotin, which is in turn contacted with the other member of the pair, such as Streptavidin-Tandem.

In accordance with a further embodiment of the invention, a kit is provided for detecting Crohn's disease in a human subject and for distinguishing Crohn's disease from ulcerative colitis and celiac sprue. The kit comprises a first antibody specific for a B cell antigen, a second antibody specific for the CD45 isoform, CD45RO, and reagent means for detecting the binding of these antibodies to B cells.

The inventors have also found that a significant proportion of first degree relatives of Crohn's disease patients have both increased intestinal permeability and an abnormal pattern of CD45 isoform expression on $CD19^+$ B-cells from PBMC in the absence of either symptoms or signs of the disease. These findings are described in Example 6.

All relatives with increased permeability had an increased fraction of $CD45RO^+$ B-cells. The percentage of relatives who were positive for CD45RO expression (approximately 10%) is the same percentage of relatives of Crohn's disease patients as have been shown by epidemiological studies to be at risk of acquiring Crohn's disease (May et al., (1993), Gastroenterology, v. 104, pp. 1627-1632).

These findings provide the basis for identifying pre-clinical Crohn's disease in relatives of patients, a group at high risk for the development of the disease, by a simple blood test. As described above with respect to detecting Crohn's disease, B cells are obtained and examined for increased expression of CD45RO. The presence of raised levels of this isoform on B cells from Crohn's disease relatives indicates a pre-clinical state and the likelihood of development of overt Crohn's disease.

MS patients have an epidemiologic link to Crohn's disease. Crohn's disease patients are 40 times more likely to acquire MS than controls and patients having both MS and Crohn's disease are more prevalent than expected given the overall incidence of these two diseases in a population.

It has now been found that a proportion of patient with MS have increased intestinal permeability when assessed by the lactulose/mannitol excretion test as described in Example 6. It has also been shown that all MS patients demonstrating increased permeability also had increased expression of CD45RO on $CD19^+$ or $CD20^+$ B-cells. In contrast, MS patients displaying normal intestinal permeability showed normal B-cell expression of CD45RO. Thees results are shown in Table 6.

In accordance with a further embodiment of the invention, a method is provided for detecting MS in a human subject by isolating or identifying peripheral B cells from the subject and examining the B cells for increased expression of the CD45 isoform, CD45RO. Suitable methods and kits for determining increased B cell expression of CD45RO have been described above.

As will be understood by those skilled in the art, although increased levels of CD45RO - positive B cells are found both in Crohn's disease and MS, the presenting symptoms of these diseases are distinguishable.

Examples

Example 1

Selection of Subjects

Specimens of colon from patients with Crohn's disease and ulcerative colitis were obtained with the assistance of a pathologist to sample disease-involved mucosa. Normal mucosa was obtained from colon at least 15 cm distal to carcinoma in patients undergoing resection as well as from normal areas in patients having resection for diverticulosis. Normal colon was also

obtained from deceased persons donating organs for transplantation. A total of 10 "normal", 8 Crohn's and 4 ulcerative colitis colons were analysed. Peripheral blood was analysed from patients with Crohn's disease (N=33), as well as patients with ulcerative colitis (N=11), celiac sprue (N=13) and normal Red Cross blood donor controls (N=9).

Many of the patients with Crohn's disease were enrolled in the MRC Canadian Crohn's Relapse Prevention Trial, and all had diagnoses established clinically and through pathologic and radiologic criteria. These patients were on various medications, but primarily 5-ASA and sulfasalazine. Patients studied were not on immunomodulatory medications.

Peripheral Blood Mononuclear Cell Isolation

Blood was obtained with informed consent from 33 patients with Crohn's disease, as well as from patients with ulcerative colitis (UC) and celiac sprue. Normal Red Cross donor blood was also obtained. Peripheral blood mononuclear cells (PBMC) were purified by centrifugation over Ficoll Paque (Pharmacia, Dorval, PQ) followed by two washes.

Isolation of Lamina Propria Lymphocytes

Normal large bowel intestinal mononuclear cells (INT MNC) were obtained from surgically removed specimens from individuals donating organs for transplantation, from patients with diverticulosis, as well as from morphologically normal areas of large bowel at least 10 cm distal to diseased areas of colons resected for adenocarcinoma. Crohn's INT MNC were obtained from colon specimens from individuals having resections done for therapeutic reasons. UC INT MNC similarly were obtained from colons of persons having therapeutic resections. Intestinal MNC were isolated from mucosa generally according to a previously described method (Bookman et al., (1979), Gastroenterology, v. 77, p. 503; MacDermott et al., (1981), Gastroenterology, v. 91, p. 379; and

MacDermott et al., (1981), Gastroenterology, v. 81, p. 844).

Briefly, specimens were washed in RPMI medium and the mucosa then dissected from the submucosa. Mucosa was then cut into small 0.5 cm X 0.5 cm minced pieces and washed repetitively in Hanks balanced salt solution (HBSS) without calcium and magnesium containing antibiotics 1 mg/ml Ticarcillin, (Beecham, Pointe-Claire, QC), 0.5 mg/ml Amikacin (Bristol Labs, Ottawa, ON), 0.4% Septra (Burroughs-Wellcome, Kirkland, QC), 2 mg/ml Fungizone Grand Island Biological Co (Gibco, Grand Island, NY), 10 mm Hepes and NaOH to adjust to pH 7.4. The minced pieces were stirred in multiple changes of medium containing 0.75 M EDTA (Sigma, St. Louis, MO) and 5% heat inactivated pooled human serum to remove epithelial cells. The tissue was then incubated overnight in HBSS-collagenase medium containing 16 mg/ml chromatographically purified collagenase (Worthington Biochemical, Freehold, NJ) and 20% heat-inactivated pooled human serum. Following collagenase digestion, cells were layered over a ficoll-hypaque gradient (S.G. 1.077) and centrifuged at 400 g for 20 minutes. The interface was collected, diluted with HBSS, resuspended in 10 ml Percoll solution (S.G. 1.040, Pharmacia, Piscataway, NJ) and centrifuged at 500 g for 15 minutes to remove dead cells and debris. These cells were then washed through fetal-bovine serum gradients and counted.

Antibodies

The CD45 common determinant marker (HLE-FITC), Leu 15-PE (CD11b) and the control antibodies IgG1-FITC, IgG, PE, IgG2a, FITC and IgG2aPE were purchased from Becton-Dickinson (Mountainview, California). B4-FITC, B4-RD1 (CD19), B1-FITC or B1-RD1 (CD20) and T1-RD1 (CD5) were purchased from Coulter (Hialeah, Florida). Biotinylated goat anti-mouse immunoglobulin and Tandem avidin were purchased from Southern Biotechnology (Birmingham, Alabama). UCHLI(CD45RO) was a generous gift

of Dr. P. Beverley. UCHLI is also available from Becton Dickinson, Inc. Monoclonal antibodies to CD45RA were CD45RAFITC, purchased from Gen Track (Wayne, Pennsylvania) and FMC44-PE (21,22) (Pilarski et al., (1989), Eur. J. Immunol., v. 19, pp. 589-597; Jensen et al., (1991), Blood, v. 78, p. 711).

Three-Color Immunofluorescence (IF)

Cell surface antigens present on the isolated MNC were evaluated by three color IF using a four stage combined direct and indirect staining procedure. In stage (i) cells were stained with an uncoupled antibody; (ii) secondly with goat anti-mouse biotin (Jackson); (iii) blocked with mouse Ig (Jackson), 1 μg/ml; and (iv) stained with Streptavidin-Tandem, together with the two remaining antibodies directly conjugated to either FITC or PE. MNC were resuspended in 50 μl of uncoupled monoclonal antibody diluted approximately in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.02% sodium azide. The cells were incubated for 30 minutes at 4°C, spun down and washed twice in buffer solution and incubated for 10 min at room temperature, spun down and resuspended in 20 μl Streptavidin-Tandem. The other two monoclonal antibodies coupled to FITC and PE were added directly and 25 μl of buffer added. This was incubated for 30 min at 4°C. Cells were washed three times and fixed with 1% formalin for flow cytometric analysis. Analysis of samples was performed on a FACScan (Becton-Dickinson). Dead cells and red cells were excluded by gating on forward angle light scatter and side scatter. All samples included staining with isotype-matched control antibodies and unstained cells. List mode files were collected of 20,000 cells from each sample, and measurements of all three fluorochromes as well as forward and side scatter were recorded.

Calculations and Statistical Methods

To determine percentages of CD3 and CD19 PBL and LPL expressing a specific marker, flow cytometry was

performed on these populations. Three-color IF data was collected in list mode from the analysis of each sample of lymphocytes. Data were then gated on the T or B-cell population of interest (CD3 or CD19) and the CD45 isoform expression of these respective populations were plotted as histograms using a Becton-Dickinson FACScan workstation using FACScan software, in comparison to an identically gated population, stained with CD3 or CD19 and isotype control monoclonal antibodies.

Of those patients with Crohn's disease whose PBL were analysed, clinical parameters including the erythrocyte sedimentation rate (ESR), disease duration, medications, disease location, age of the patient and disease severity (presence or absence of fistulae or extraintestinal manifestations of disease) as well as the Crohn's disease activity index (CDAI) were obtained from chart review. The CDAI is a detailed index of Crohn's disease activity as measured by eight selected variables including number of stools, abdominal pain, general well-being, the need for opiates or related antimotility medications, abdominal masses, hematocrit, and body weight (Streuli et al., supra). Multiple regression analysis was performed by SPSS/PC+ using these clinical variables with high or low molecular mass isoforms (CD45RA and/or CD45RO) CD45 and the combination of its isoforms as dependent variables. The students T-test was used to compare CD45 isoform expression on T or B-cells between types of disease.

Predominant Expression of CD45RO on $CD3^+$ T-cells of Mucosal LPL from Normal, Crohn's and Ulcerative Colitis Patients

Lamina propria lymphocytes isolated from fresh intestinal mucosa were analysed from normal, Crohn's disease and UC tissue. Normal mucosa was derived from patients with diverticular disease, areas distal to neoplasms and deceased organ donors.

CD45 isoform staining (CD45RA and RO) on $CD3^+$ lamina propria lymphocyte T-cells was detected by three color immunofluorescence. CD45RA was detected by FMC44PE. CD45RO was detected by UCHL1 and indirectly stained with biotinylated goat anti-mouse immunoglobin followed by Tandem-avidin, and CD3 was detected by Leu4-FITC. Files of 20,000 cells were electronically gated to include only $CD3^+$ cells and dot plots of CD45RA versus CD45RO staining generated. The number of positive cells in each quandrant was determined in comparison to identically gated samples stained with Leu4-FITC, IgG1PE and IgG2a biotinylated goat anti-mouse Ig/Tandem avidin. Results are shown in Table 1, values being reported as mean ± standard error.

Heterogeneity of CD45 Isoform Expression on $CD3^+$ T Peripheral Blood Lymphocytes From Normal, Crohn's and Ulcerative Colitis Patients

CD45 isoform staining (CD45RA and RO) on $CD3^+$ T-cells was detected by three color immunofluorescence as described in Example 1.

Normal PBMC were analysed, files were gated on $CD3^+$ and CD45 isoform expression was quantitated. No abnormalities in proportion of CD3, CD4 or CD8 cells were detected, compared with previous reports.

Results are shown in Table 2 (mean ± SE).

Example 2

Analysis of CD45 Isoforms of $CD19^+$ BLPL From Normal, Crohn's and UC Patients

CD19 LPL from IBD and from normal patients (organ donor, diverticulosis and neoplasm resections) were analysed to determine expression of CD45RA and RO isoforms. CD45 isoform staining (CD45RA and RO) on $CD19^+$ or $CD20^+$ B-cell lamina propria lymphocytes was detected by three color immunofluorescence. CD45RA was detected by FMC44PE. CD45RO was detected by UCHLI and stained indirectly with biotinylated goat anti-mouse

immunoglobulin and Tandem-avidin, and CD19 by B4-FITC, or CD20 by B1-FITC. Similar results were obtained using either CD19 or CD20. Files were gated for $CD19^+$ cells and CD45 isoform expression as described in Example 1. The mean percentage of $CD19^+$ B-cells in each disease studied were: normal blood donors mean=8% ± 0.5%, Crohn's disease mean=10% ± 5%, UC mean=8% ± 5%. Results are shown in Table 3 (mean ± SE).

Example 3

Increased Proportion of Late Stage $CD45RO^+$ B-cells in $CD19^+$ B PBMC From Crohn's, As Compared to Normal Donors or Celiac Sprue and UC Patients

PBMC from Crohn's patients with detailed clinical descriptions were analysed in comparison to normal donors. CD45 isoform staining (CD45RA and RO) on $CD19^+$ B-cell peripheral blood mononuclear cells was detected by three color immunofluorescence as described in Example 2.

Results are shown in Table 4 (mean ± SE).

Example 4

$CD19^+$ B PBL From Normal and Crohn's Patients are CD11b- and mainly CD5-

CD5 and CD11b expression on $CD19^+$ B-cell PBMC from patients with Crohn's disease was analysed. No CD11b was found on $CD19^+$ B-cell PBMC from patients with Crohn's disease, or on B-cells from normal donors. A low level of CD5 expression (mean 7% ± 1%, N=19) was found on $CD19^+$ B PBL in Crohn's patients, less than that found on B-cells from normal donors (mean=30% ± 5%, N=7) (data not shown). Since CD5 and CD11b have been shown to increase on the activation of B-cells, the lack of either antigen on the B-cells argues against any activating effect of the procedures used to isolate them.

Example 5

Correlation Between the Proportion of CD45RO$^+$ B-cells and Severity of Crohn's Disease

Review of the expression of CD45RA or RO in CD19$^+$ PBMC from individual patients with Crohn's disease showed considerable individual variation, in clear contrast to the normal pattern, as shown in Figure 3. To assess the clinical significance of this marked variation in CD45RA and RO expression on CD19$^+$ B-cells, clinical data were obtained on each patient. A number of parameters known to be associated with Crohn's clinical disease severity were tabulated, and multiple regression analysis was performed on these values using the statistical program SPSS/PC+. By using multiple regression analysis with the B-cell subsets defined by CD45 isoforms as dependent variables and the clinical parameters (Crohn's disease activity index (CDAI), disease duration, erythrocyte sedimentation (ESR), medications, age of patient and disease severity) as independent variables, a statistically significant relationship was identified. Two systems of analysis were incorporated into the multiple regression analysis. Firstly, CD45RO$^+$ B-cells were divided into 2 groups, group 1 included B-cells with expressing 0-49% CD45RO$^+$. Group 2 included all patients with B-cells having 50% and greater CD45RO$^+$ expression. These values were compared with the respective CDAI values for each patient, showing the means of the two groups to be significantly different using the two-sample t test (group 1 mean=108.2, se=16.5) (group 2 mean=179.0, se=27.4) (n_1=23, n_2=6, t=2.01, 2 tail p=0.05). Moreover, evaluation of ungrouped percentages of CD19$^+$ B-cells expressing CD45RO$^+$ revealed a significant correlation with CDAI (coefficient=0.303, p=0.05). The correlation between CD45RA$^-$RO$^+$ CD19$^+$ PBMC and the CDAI is linear, with increased severity of disease (high CDAI) associated with increased numbers of

$CD45RA^-RO^+$ $CD19^+$ B-cells. Furthermore, there is a significant correlation of high levels of $CD45RA^-RO^+$ $CD19^+$ with high CDAI values, as shown in Figure 4. No other combination of parameters or any B-cell phenotype had such a significant relationship.

All CD45 isoform values were evaluated as dependent values using multiple regression analysis with the independent values (Crohn's disease activity index (CDAI), disease duration, medication, erythrocyte sedimentation rate (ESR), Crohn's disease location, and patient age). Regression analysis was performed to account for the marked range of CD45RO expression on $CD19^+$ cells among patients with Crohn's disease. A statistically significant relationship was identified between $CD45RA^-RO^+$ expression on $CD19^+$ cells and the clinical parameter CDAI correlation coefficient=0.303 ($p=0.05$). Furthermore, if patients were divided into two groups such that group 1 CDAI <50, group 2 CDAI 50+, the mean $CD45RO^+$ expression on $CD19^+$ B-cells for group 2 was 75% higher than group 1, a clinically and statistically significant finding ($n_1=23$, $n_2=6$, $t=2.01$, 2 tail $p=0.05$, $se=27.4$). The outlying point with 60% $CD45RO^+$ $CD19^+$ B-cells therefore is statistically acceptable since this patient had a CDAI >50.

Example 6

Intestinal permeability was examined in fifteen patients with Crohn's disease, as determined by standard clinical criteria, in thirteen of their first degree relatives and in ten control volunteers of similar age.

All relatives and controls were entirely free of gastrointestinal symptoms, had no history of renal disease or diabetes and had not taken nonsteroidal anti-inflammatory medications or alcohol for at least two weeks prior to the study. The same drug and alcohol

exclusion criteria were also applied to the patient population.

Intestinal Permeability Study Protocol:

After an overnight fast, all participants drank a 250 ml solution containing 5 g lactulose, 2 g mannitol and 5 g glucose made isosmotic with NaCl. All chemicals were purchased from Sigma (St. Louis, MO) and were of the highest grade available. Urine was collected for five hours into pre-weighed containers to which 7.5 ml of 10% thymol had been added as a preservative. During the urine collection, the subjects were only allowed to drink water, black coffee or tea. Urine was assayed immediately for sugar concentrations by high performance liquid chromatography (HPLC) as described in May et al. (1993), Gastroenterology, v. 104, pp. 1627-1632. Immediately upon completion of the urine collection, 10 ml of peripheral blood was obtained from each participant. PBMC were purified by centrifugation over Ficoll Paque (Pharmacia, Dorval, PQ) followed by two washes.

Antibodies:

The CD45 common determinant marker (HLE-FITC) and the control antibodies IgG1-FITC, IgG1-PE, IgG2a-FITC and IgG2a-PE were purchased from Becton-Dickinson (Mountainview, California). β-FITC, β-RD1 (CD19), β-FITC or β-RD1 (CD20) were purchased from Coulter (Hialeah, Florida). Biotinylated goat anti-mouse immunoglobin and Tandem Avidin were purchased from Southern Biotechnology (Birmingham, Alabama), UCHL1 (CD45RO) was a generous gift of Dr. P. Beverley. Monoclonal antibodies to CD45RA were CD45RA-FITC and were purchased from Gen Track (Wayne, Pennsylvania). The specificity of CD45 antibodies used was confirmed by their appropriate molecular weights and all were tested for their reactivity with a panel of CD45 transfectants.

Three Color Immunofluorescence:

Cell surface antigens on the isolated MNC were evaluated by three color immunofluorescence as described in Example 1.

Analysis of Lactulose and Mannitol:

Urine samples (10 ml) were deionized by adding 1 g of a 1:15 (wt:wt) mixture of Amberlite IR-120 and IRA-400 resin (BDH Chemicals, Toronto, Ontario, Canada). Sucrose was added as an internal standard, and the supernatant filtered through a 40 μm millepore filter (Millepore, Bedford, MA). Samples were separated on a Hamilton RCX-10 anion exchange column (Reno, NV) in a HP 1090 HPLC (Hewlett Packard, Toronto, Ontario, Canada) at room temperature using 30 mmol/L NaoH as the isocratic mobile phase. Peak identification was accomplished with the use of authentic standards and detected using pulsed amperometric electrochemical detection on a gold electrode. Samples were diluted as necessary after addition of the internal standard. Quantitation was performed using known standards at multiple concentrations with linear interpolation between concentrations. All samples were diluted so that concentrations of interest fell within the range of standards. Data were expressed as the fractional excretion of lactulose or mannitol and the lactulose/mannitol ratio calculated directly from these numbers.

Statistics:

The data acquired (% CD45RO $CD19^+$ B-cell expression and intestinal permeability Lac/Man ratio) were compared using SAS (Statistical Analysis Systems, SAS Inc., North Carolina) and analysis of variance.

Table 5 shows the location of Crohn's disease in the fifteen patients studied, determined by

radiological or endoscopic methods. Previous therapies are also shown.

Intestinal Permeability:

Figure 6 illustrates the lactulose/mannitol ratio found in all study groups. A group of historical controls (n=40) is included and these represent an ongoing control group studied in the laboratory of the inventors as internal controls. None of the subjects were taking non-steroidal inflammatory drugs or ethanol for the week prior to testing. The upper limit of normal (defined as the mean of this group plus 2STD) is shown by the dotted line in Figure 5. Ratios above 0.022 are considered abnormal. Controls used in this study (current controls) had lactulose/mannitol ratios uniformly within this normal range. In contrast, a significant proportion of patients with Crohn's disease had increased intestinal permeability, some with very high values. Ten of the 15 patients (67%) had high permeability while 5 fell within our normal range. Seven of 13 relatives (54%) also demonstrated high permeability in the absence of either symptoms or signs of disease.

Immunological Alterations:

In Figure 6, the percentage of peripheral $CD19^+$ B-cells bearing the CD45RO isoform is illustrated for both controls and patients with Crohn's disease. In all 10 controls, less than 6% of peripheral B-cells were found to be positive for this isoform. This value was, therefore, used as the upper limit of normal and is represented by the dotted line in Figure 6.

Figure 6 patients are grouped as having either normal permeability or high permeability based on the data shown in Figure 5. Of the 10 patients with high permeability, 8 demonstrated an abnormally high fraction of B-cells positive for CD45RO. This situation was found in only 2 of the 5 patients with normal permeability.

Since this observation could be related to disease activity in these patients rather than the permeability defect *per se*, this relationship was examined in the relative population. These data are shown in Figure 7. The same control data is shown but now the fraction of CD45RO positive peripheral B-cells is shown for the relatives of Crohn's disease patients. Once again, the relatives were grouped into those with normal or high intestinal permeability, based on the data from Figure 5. All relatives with normal permeability had a normal fraction of CD45RO positive B-cells (up to about 4-5%), whereas all relatives with increased permeability demonstrated an increased fraction of CD45RO positive B-cells.

Example 7

Six subjects diagnosed by a neurologist, by standard clinical criteria, as having multiple sclerosis were examined for intestinal permeability, as described in Example 6, and for increased B cell expression of CD45RO, as described in Example 1. The results are shown in Table 6.

Of the six subjects, two (SMSLM and SMSPK) were in apparent remission and had normal intestinal permeability and only slightly raised B cell CD45RO expression. The other sour subkjects, not in remission, showed greatly raised CD45RO expression and B cells and the two examined showed raised intestinal permeability.

TABLE 1

$CD3^+$ MUCOSAL T CELLS

	$CD45RA^+RO^-$	RA^+RO^+	RA^-RO^+	$RA^- RO^-$
CROHN'S (N=8)	23% ± 6%*	20% ± 6%	56% ± 9%	0
UC (N=4)	22% ± 9%*	9% ± 5%*	57% ± 18%	12% ± 11%
NORMAL (N=10)	9% ± 2%*	19% ± 4%	66% ± 8%	6% ± 4%

*$p<0.05$ versus normal

p--all other pairs not significant

TABLE 2

$CD3^+$ PERIPHERAL BLOOD T CELLS

	$CD45RA^+RO^-$	RA^+RO^+	RA^-RO^+	RA^-RO^-
CROHN'S (N=21)	34% ± 5%	10% ± 2%	37% ± 5%	16% ± 4%*
UC (N=11)	44% ± 3%	19% ± 3%	37% ± 4%	0*
CELIAC (N=13)	41% ± 4%	9% ± 2%	44% ± 6%*	4% ± 3%*
NORMAL (N=7)	44% ± 6%	9% ± 3%	42% ± 3%	1% ± 0*

* $p<0.05$ Crohn's versus normal, Crohn's versus UC, Crohn's versus celiac sprue.

p--all other pairs not significant

TABLE 3

$CD19^+$ MUCOSAL LYMPHOCYTES

CD45	RA^+RO^-	RA^+RO^+	RA^-RO^+	RA^-RO^-
CROHN'S (N=6)	40% ± 8%	21% ± 7%	37% ± 10%	2% ± 1%
UC (N=4)	45% ± 13%	26% ± 5%	29% ± 16%	0% ± 1%
NORMAL (N=10)	45% ± 5%	27% ± 4%	23% ± 6%	5% ± 2%

p value between all pairs of data-not significant

TABLE 4

CD19$^+$ PERIPHERAL BLOOD MONONUCLEAR CELLS

CD45	RA^+RO^-	RA^+RO^+	RA^-RO^+	RA^-RO^-
CROHN'S (N=17)	58% ± 9%*	13% ± 4%+	15% ± 5%+	16% ± 5%+
UC (N=11)	98% ± 0%*	2% ± 0+	8% ± 0%+	0+
CELIAC (N=11)	96% ± 1%*	2% ± 1%+	1% ± 0+	0+
NORMAL (N=9)	99% ± 1%*	0*	0*	0*

* $p<0.005$ Crohn's versus normal, Crohn's versus UC, Crohn's versus celiac sprue.

+ $p<0.05$ Crohn's versus UC and Crohn's versus celiac sprue.

TABLE 5

Parameter	Number (%)
Disease Location	
Small Intestine	7(47)
Large Intestine	6(40)
Both	2(13)
Previous Therapy	
5-ASA Products	8(53)
Steroids	1(7)
Both	4(27)
Neither	2(13)

TABLE 6

Subject Identification	Lactulose/Mannitol Excretion Ratio	% B cells expressing CD45RO
SMSEM	0.050	94
SMSTM	0.025	40
SMSLM	0.015	10
SMSPK	0.010	9
SMSKH		18
SMSLK		58

CLAIMS:

1. A method for detecting Crohn's disease in a human subject comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of said antigen being indicative of Crohn's disease.

2. The method of claim 1, wherein peripheral blood mononuclear cells are purified from said blood sample; and

said peripheral blood mononuclear cells are contacted with a first antibody bearing a first detectable label, the antibody being specific for a B cell antigen, whereby the B cells are identified, and with a second antibody bearing a second label detectable separately from the first label, the second antibody being specific for CD45RO.

3. A method for distinguishing Crohn's disease from ulcerative colitis and celiac sprue in a human subject presenting with intestinal symptoms, comprising

obtaining a peripheral blood sample from the subject;

isolating or identifying B cells from the blood sample; and

determining the antigen CD45RO on the surface of the B cells,

the presence of said antigen being indicative of Crohn's disease.

4. A method for detecting pre-clinical Crohn's disease in a human subject, comprising

obtaining a peripheral blood sample from the subject;
isolating or identifying B cells from the blood sample; and
determining the antigen CD45RO on the surface of the B cells,

the presence of said antigen being indicative of pre-clinical Crohn's disease.

5. A method for detecting multiple sclerosis or pre-clinical multiple sclerosis in a human subject, comprising

obtaining a peripheral blood sample from the subject;
isolating or identifying B cells from the blood sample; and
determining the antigen CD45RO on the surface of the B cells,

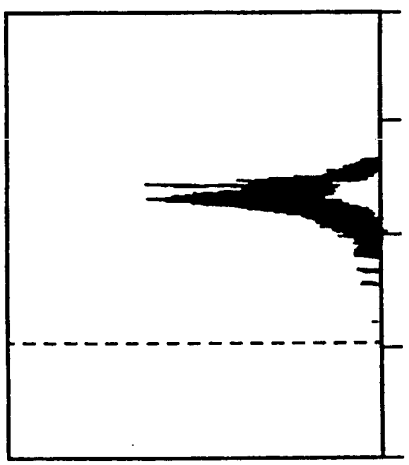
the presence of said antigen being indicative of multiple sclerosis or pre-clinical multiple sclerosis.

6. A kit for detecting Crohn's disease or pre-clinical Crohn's disease in a human subject, comprising

(a) a first antibody specific for a B cell antigen;
(b) a second antibody specific for the CD45 isoform, CD45RO; and
(c) reagent means for detecting the binding of said antibodies to peripheral B cells.

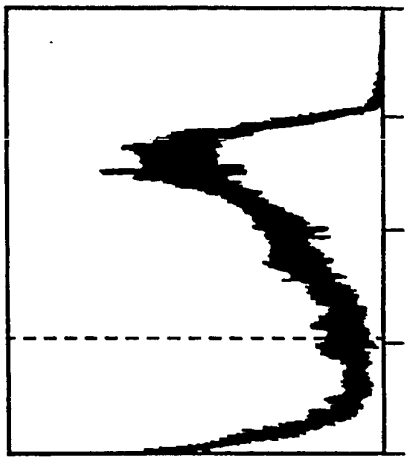
7. A kit in accordance with claim 6 wherein the first antibody bears a first detectable label and wherein the second antibody bears a second label separately detectable from said first label.

8. A kit in accordance with claim 7 wherein the first antibody is an anti-CD19 or anti-CD20 antibody.



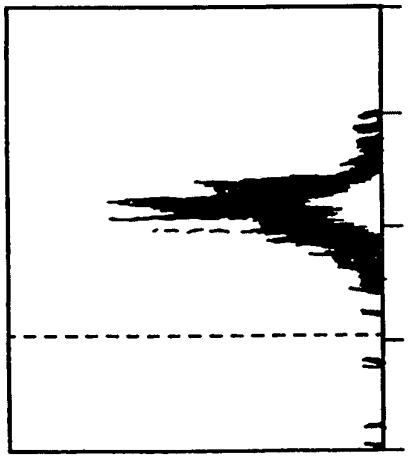
ULCERATIVE COLITIS

FIG.1B.



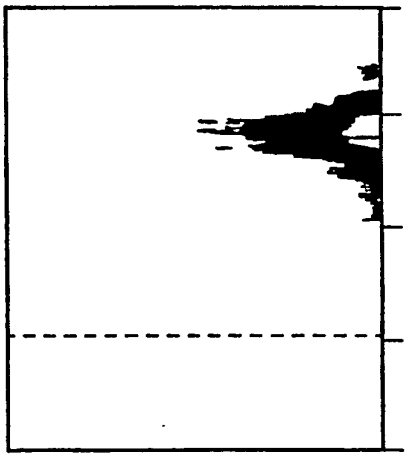
CROHN'S

FIG.1D.



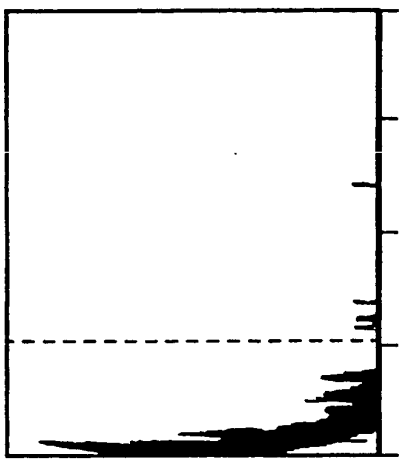
NORMAL

FIG.1A.



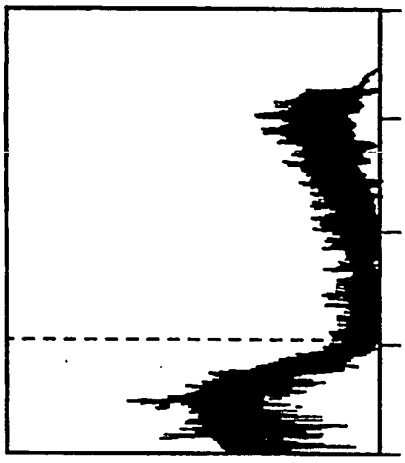
CELIAC SPRUE

FIG.1C.



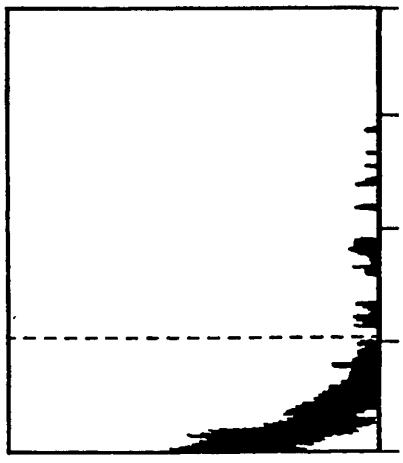
ULCERATIVE COLITIS

FIG.2B.



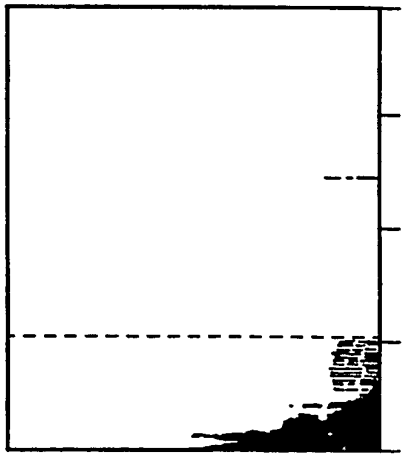
CROHN'S

FIG.2D.



NORMAL

FIG.2A.



CELIAC SPRUE

FIG.2C.

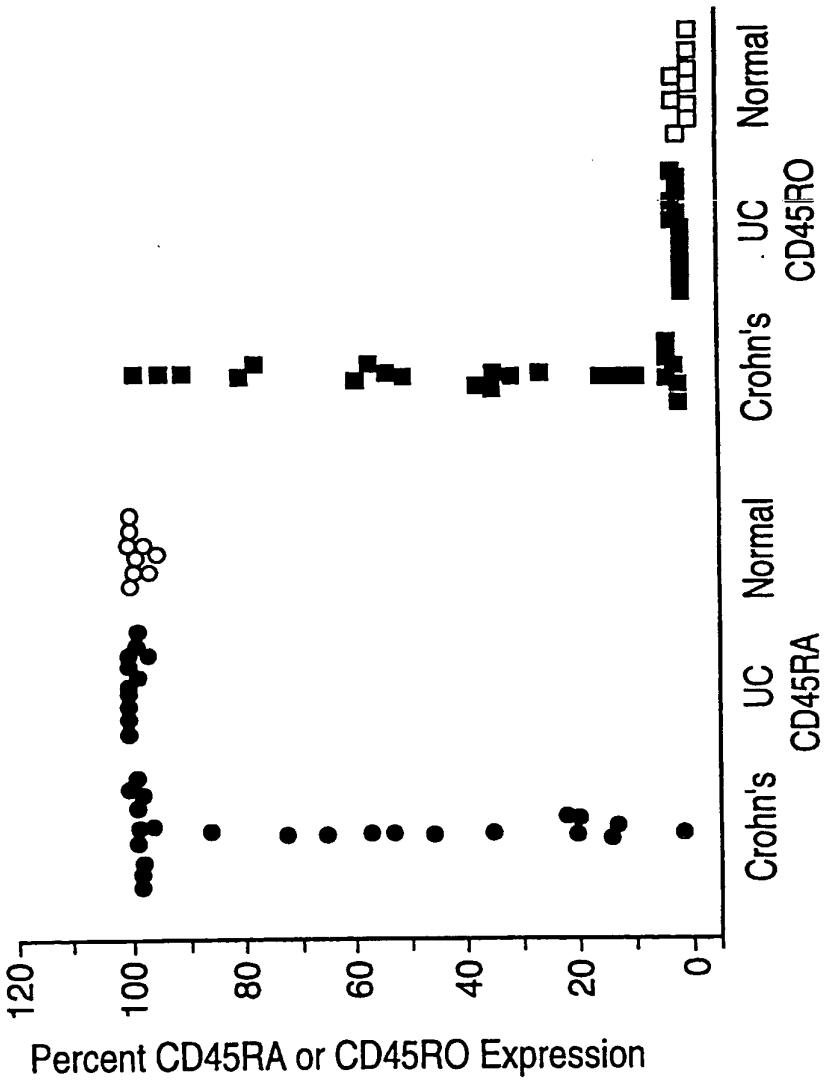

Crohn's UC CD45RA
Normal
Crohn's UC CD45RO
Normal
120
100
80
60
40
20
0
Percent CD45RA or CD45RO Expression

FIG.3.

CDAI VS TOTAL CD45RO+

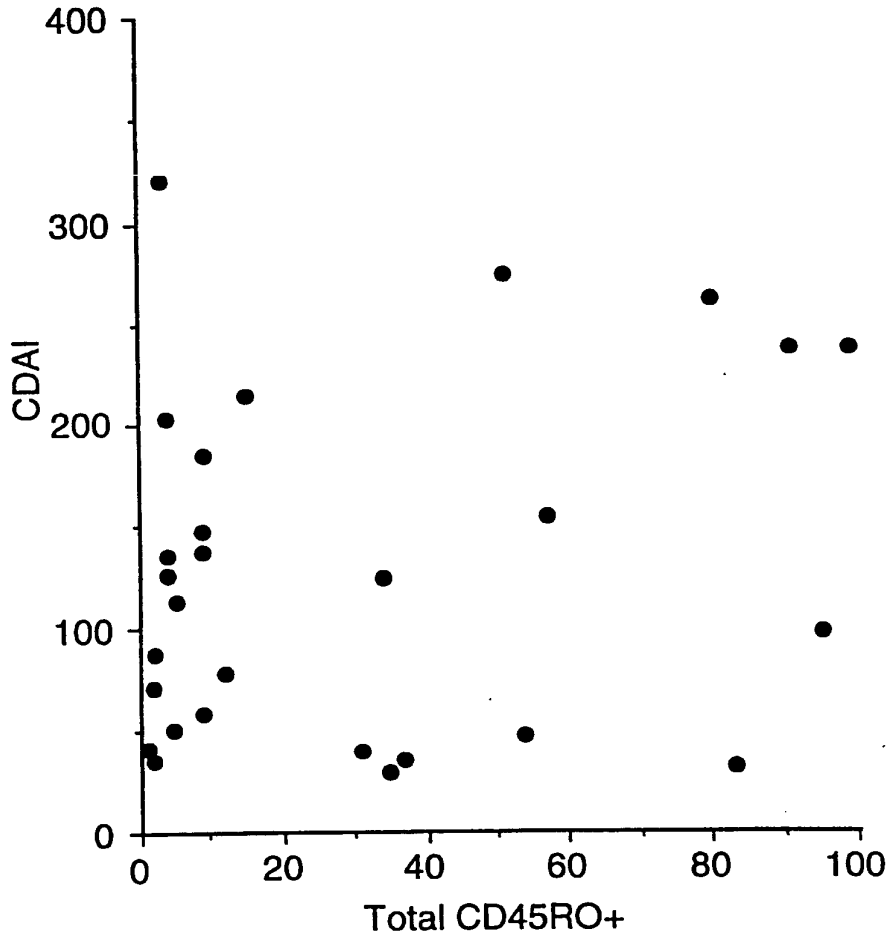


FIG.4.

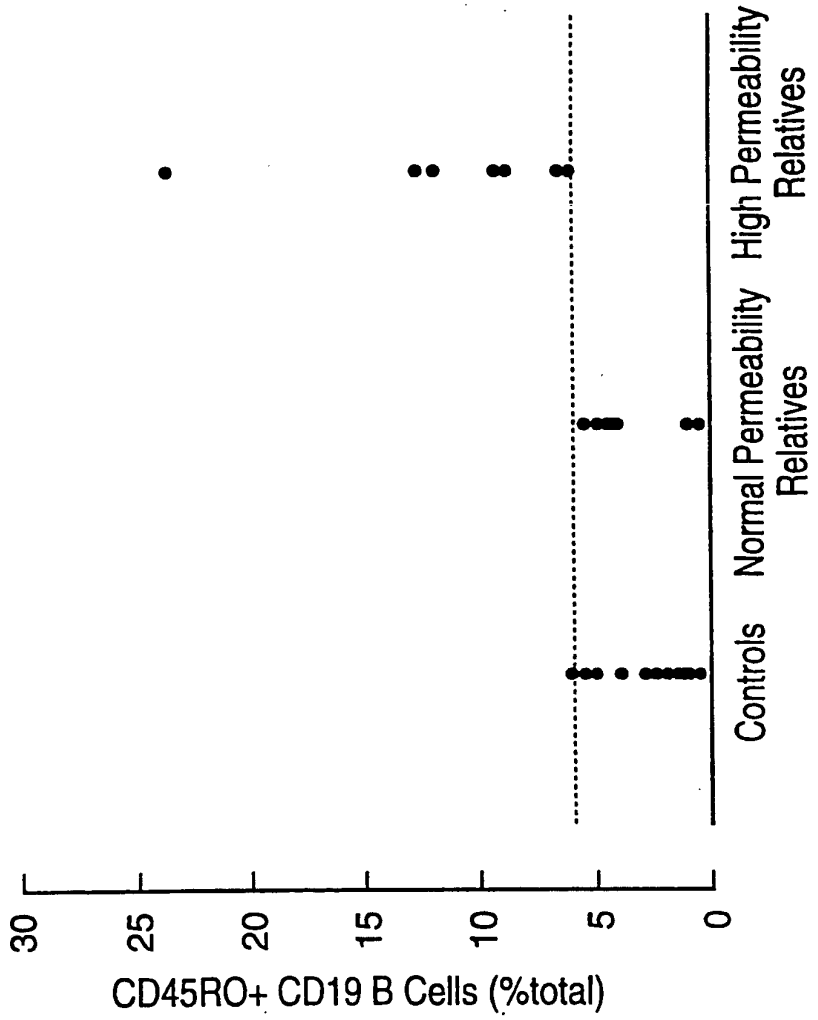

Controls
Normal Permeability Relatives
High Permeability Relatives
30
25
20
15
10
5
0
CD45RO+ CD19 B Cells (%total)

FIG.5.

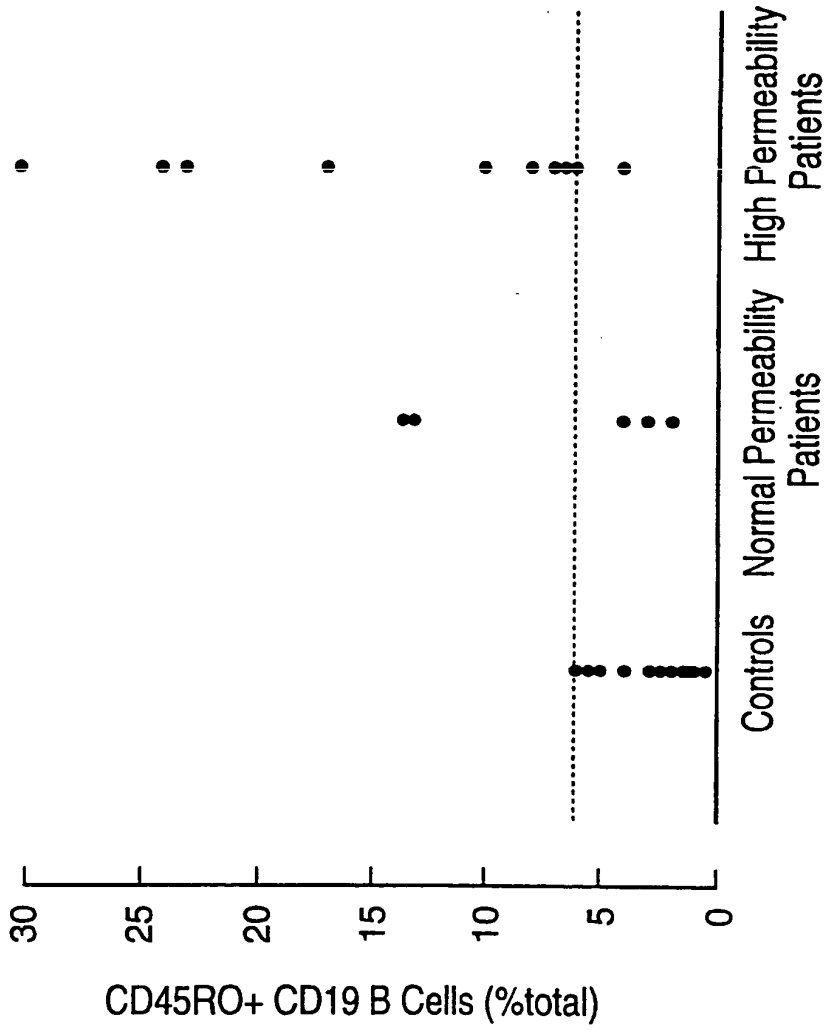

Controls
Normal Permeability Patients
High Permeability Patients
30
25
20
15
10
5
0
CD45RO+ CD19 B Cells (%total)

FIG.6.

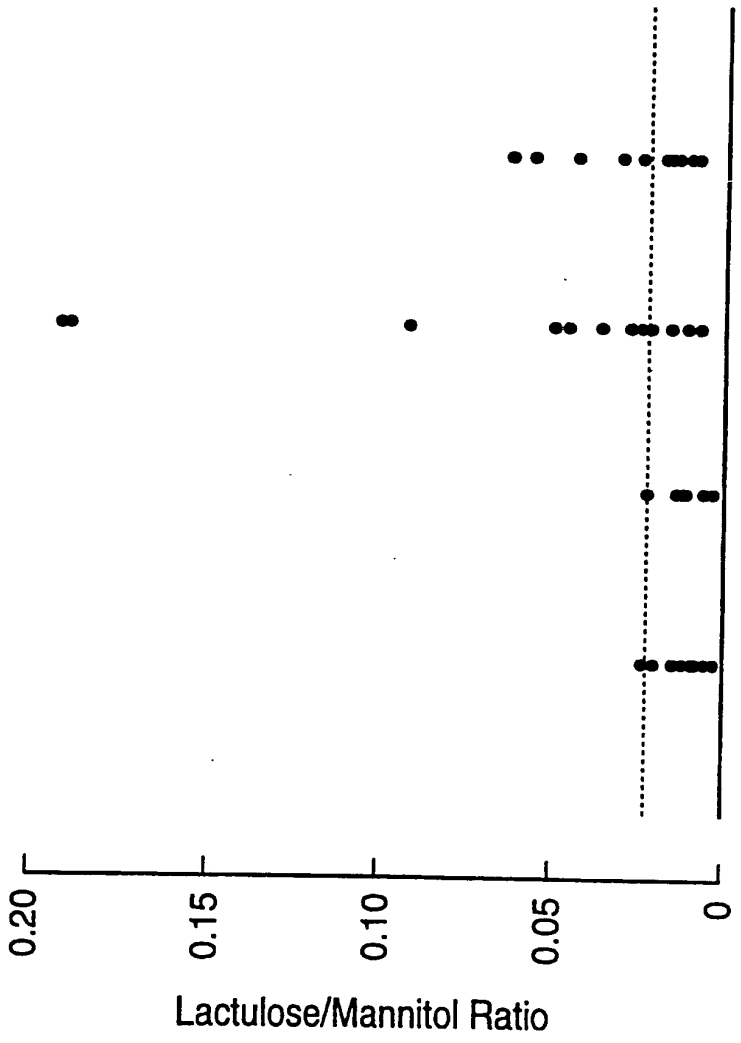

Historical Controls
Current Controls
Patients
Relatives
0.20
0.15
0.10
0.05
0
Lactulose/Mannitol Ratio

FIG.7.

INTERNATIONAL SEARCH REPORT

Inten nal Application No
PCT/CA 94/00635

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/564 G01N33/58 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GUT 34 (12). 1993. 1698-1704. ISSN: 0017-5749 Yacyshyn B R et al 'Expression of CD45RO on circulating CD19+ B-cells in ohn 's disease.' see the whole document ---	1-4,6-8
P,X	95TH ANNUAL MEETING OF THE AMERICAN GASTROENTEROLOGICAL ASSOCIATION, NEW ORLEANS, LOUISIANA, USA, MAY 15-18, 1994. GASTROENTEROLOGY 106 (4 SUPPL.). 1994. A280. ISSN: 0016-5085 Yacyshyn B R et al 'Increased intestinal permeability appears to correlate with the peripheral blood CD19+ B - cell CD45RO + phenotype.' see the whole document --- -/--	1,3,4

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1995	05. 04. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Döpfer, K-P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 94/00635

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUT, vol.28, no.6, June 1987, LONDON,GB pages 745 - 753 F.PALLONE, S.FAIS, O. SQUARCIA, L.BIANCONE, P.POZILLI AND M.BOIRIVANT 'Activation of peripheral blood and intestinal lamina propria lymphocytes in Crohn's disease. In vivo state of activation an in vitro response to stimulation as defined by the expresssion of early activation antigens' cited in the application see the whole document	1-4,6-8
A	INTERNATIONAL IMMUNOLOGY, vol.1, no.3, July 1989, OXFORD, GB pages 229 - 236 GITTE S. JENSEN ET AL. 'Transition in CD45 isoform expression during differentiation of normal an abnormal B cells' cited in the application see the whole document	1-8
A	AMERICAN JOURNAL OF HEMATOLOGY, vol.37, no.1, May 1991, NEW YORK, NY, US pages 20 - 30 GITTE S. JENSEN ET AL. 'Transitions in CD45 Isoform Expression Indicate Continuous Differentiation of a Monoclonal CD5+ CD11+ B Lineage in Waldenstrom's Macroglobulinemia' cited in the application see page 21, right column, line 25 - page 22, left column, line 31 see page 25, left column, line 6 - page 26, left column, line 8; figures 4,5,6	6-8
A	DIG DIS SCI 36 (10). 1991. 1453-1458. CODEN: DDSCDJ ISSN: 0163-2116 SENJU M ET AL 'TWO-COLOR IMMUNOFLUORESCENCE AND FLOW CYTOMETRIC ANALYSIS OF MINA PROPRIA LYMPHOCYTE SUBSETS IN ULCERATIVE COLITIS AND CROHN 'S DISEASE.'	
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